METHOD FOR PRODUCING L-LYSINE OR L-ARGININE BY USING METHANOL-ASSIMILATING BACTERIUM

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to techniques useful in the microbial industry. More specifically, the present invention relates to a method for producing L-lysine or L-arginine by fermentation, and a microorganism used in the production method.

Background Art

[0002] Amino acids such as L-lysine, L-glutamic acid, L-threonine, L-leucine, L-isoleucine, L-valine and L-phenylalanine are industrially produced by fermentation using microorganisms that belong to the genus *Brevibacterium* (*Corynebacterium*), *Bacillus*, *Escherichia*, *Streptomyces*, *Pseudomonas*, *Arthrobacter*, *Serratia*, *Penicillium*, *Candida* or the like. Strains isolated from nature, or artificial mutants thereof, have been used to improve the productivity of these microorganisms. Moreover, various techniques have been disclosed for increasing the L-amino acid producing abilities, such as recombinant DNA techniques to enhance L-amino acid biosynthetic enzymes.

[0003] Production of L-amino acids has been considerably increased by breeding of microorganisms such as those mentioned above with improved production methods. However, in order to respond to further increases in demand in future, development of methods which provide more efficient production of L-amino acids at lower cost are clearly still necessary, and therefore, still represent a need in the art.

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[0004] Methanol is a known fermentation raw material which is available in large amounts at a low cost. Methods for producing L-amino acids by fermentation using methanol are known, and include methods using microorganisms that belong to the genus *Achromobacter* or *Pseudomonas* (Japanese Patent Laid-open (Kokai) No. 45-25273), *Protaminobacter* (Japanese Patent Publication (Kokoku) No. 49-125590), *Protaminobacter* or *Methanomonas* (Japanese Patent Laid-open No. 50-25790), *Microcyclus* (Japanese Patent Laid-open No. 52-18886), *Methylobacillus* (Japanese Patent Laid-open No. 4-91793), *Bacillus* (Japanese Patent Laid-open No. 3-505284) and so forth. The inventors of the present invention have developed methods for producing L-amino acids by breeding *Methylophilus* bacteria using artificial mutagenesis and recombinant DNA techniques (WO 00/61723).

[0005] In recent years, proteins have been identified that have a function of specifically secreting an L-amino acid to the outside a cell of microorganism, as well as genes which encode these proteins. In particular, Vrljic et al. have identified a gene involved in secretion of L-lysine derived from *Corynebacterium glutamicum* R127 to the outside of a cell (Vrljic M., Sahm H., Eggeling L., Molecular Microbiology 22:815-826 (1996)). This gene was designated as *lysE*, and it was reported that L-lysine producing ability of *Corynebacterium* bacteria could be improved by enhancing the expression of this gene in *Corynebacterium* bacteria (WO97/23597). The gene *lysE* is known to secrete not only L-lysine, but also L-arginine (Bellmann A., Vrljic M., Patek M., Sahm H., Kramer R., Eggeling L. Microbiology, 147:1765-1774 (2001)). It is also known that production of some L-amino acids can be improved by increasing expression amounts of amino acid secreting proteins in *Escherichia coli* (Japanese Patent Laid-open No. 2000-189180). For example, it is reported that production of cysteine, cysteine and so forth can be

improved by enhancing the expression of ORF306 gene in *Escherichia coli* (EP885962).

[0006] However, there have been no reports to date suggesting that the amino acid secretion process is involved either positively or negatively in amino acid production by fermentation of methanol using a methanol-assimilating bacterium.   There have also been no reports suggesting an amino acid secretion gene that can provide secretion activity in a methanol-assimilating bacterium.

SUMMARY OF THE INVENTION

[0007] An object of the present invention is to provide a method for efficiently producing L-lysine or L-arginine using methanol, which is abundantly and inexpensively available.

[0008] It is a further object of the present invention to provide a bacterium belonging to the genus *Methylobacillus*, into which a DNA which is able to be expressed is introduced, and said bacterium having an ability to produce L-lysine or L-arginine, wherein said DNA encodes a variant of a protein, the protein having a loop region and six hydrophobic helixes and is involved in secretion of L-lysine to the outside of a cell, and wherein said variant does not contain said loop region and facilitates secretion of L-lysine, L-arginine or both to the outside of a methanol-assimilating bacterium when said DNA is introduced into said methanol-assimilating bacterium.

[0009] It is even a further object of the present invention to provide the bacterium as described above, wherein said mutant protein substantially consists of only the hydrophobic helixes.

[0010] It is even a further object of the present invention to provide the bacterium as described above, wherein said variant has six hydrophobic helixes.

[0011] It is even a further object of the present invention to provide the bacterium as

described above, wherein said variant is a complex comprising a peptide containing the first, second, and third hydrophobic helixes relative to the N-terminus, and a peptide containing the fourth, fifth, and sixth hydrophobic helixes relative to the N-terminus.

[0012] It is even a further object of the present invention to provide the bacterium as described above, wherein the protein is LysE protein.

[0013] It is even a further object of the present invention to provide the bacterium as described above, wherein said LysE protein is derived from a coryneform bacterium.

[0014] It is even a further object of the present invention to provide a bacterium belonging to the genus *Methylobacillus*, into which a DNA which is able to be expressed is introduced, and which has an ability to produce L-lysine or L-arginine, wherein said DNA encodes a protein selected from the group consisting of:

(A) a protein which comprises the amino acid sequence of SEQ ID NO: 10, and

(B) a protein which comprises the amino acid sequence of SEQ ID NO: 10 including substitution, deletion, insertion or addition of one or several amino acid residues, and wherein said protein shows an activity for facilitating secretion of L-lysine, L-arginine or both to the outside of a methanol-assimilating bacterium.

[0015] It is even a further object of the present invention to provide a method for producing L-lysine or L-arginine, comprising culturing the bacterium belonging to the genus *Methylobacillus* as described above in a medium to produce and accumulate L-lysine or L-arginine in culture, and collecting L-lysine or L-arginine from the culture.

[0016] It is even a further object of the present invention to provide the method for producing L-lysine or L-arginine as described above, wherein the medium contains methanol as a main carbon source.

[0017] According to the present invention, L-amino acid production, especially L-lysine

and L-arginine, using a methanol-assimilating bacterium can be improved.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Fig. 1 shows constructions of a plasmid pRStac having the tac promoter and plasmids pRS*lysE* and pRS*lysE*24 consisting of the plasmid pRStac inserted with the *lysE* gene or *lysE*24 gene.

[0019] Fig. 2 shows construction of a plasmid pRS*lysE*dapA having the *lysE*24 gene and dapA* gene.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0020] The inventors of the present invention assiduously studied in order to achieve the aforementioned objects. Initially, they found L-amino acid production by methanol-assimilating bacterium, especially *Methylobacillus* bacterium, was not possible due to failure of the L-amino acid secretion process to the outside of cells. Then, they successfully isolated a gene that provided amino acid secretion activity, especially in the microorganism, which aided in enabling efficient amino acid production.

[0021] The inventors of the present invention introduced a gene derived from a *Brevibacterium lactofermentum* 2256 strain , which is a homologue of the known *lysE* gene from a *Corynebacterium glutamicum* R127, into a methanol-assimilating bacterium and investigated its effect on amino acid production.

[0022] It shoud be noted that bacteria which was classified into the genus *Brevibacterium* has been united into the genus *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255 (1981)).

[0023] It was found that introduction of the *lysE* gene into a methanol-assimilating bacterium resulted in a mutation or deletion, and thus LysE protein could not function.

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Proteins responsible for secretion typically need to be incorporated into the cell membrane in order to function, therefore, the protein and membrane conditions such as lipid composition must be suitable for each other. It was concluded that it would be difficult to express a heterologous membrane protein, such as LysE, so that the protein can function, and this conclusion was supported by the aforementioned result.

[0024] Therefore, the inventors of the present invention found a mutant gene that could function in a methanol-assimilating bacterium while researching the aforementioned L-amino acid secretion genes. Furthermore, they found a marked effect upon use of this mutant gene in amino acid production using a methanol-assimilating bacterium.

[0025] Hereinafter, the present invention will be explained in detail.

DNA of the Present Invention

[0026] The DNA of the present invention is a DNA that promotes secretion of L-lysine, L-arginine or both to the outside of a cell when it is introduced into a methanol-assimilating bacterium, and it is a DNA that encodes a variant of a protein which is involved in the secretion of L-lysine to the outside of a cell of the microorganism.

[0027] In the present invention, the expression "facilitating secretion of L-lysine, L-arginine or both to the outside of a cell" means that when a methanol-assimilating bacterium containing the DNA of the present invention is cultured in a medium, it provides an increased amount of L-lysine, L-arginine or both secreted into the medium compared with the methanol-assimilating bacterium not containing the DNA of the present invention. The increased secretion of the L-amino acids from the inside to the outside of the cell is demonstrated by increasing L-amino acid accumulation in the medium during the culture of the methanol-assimilating bacterium containing the DNA

of the present invention as compared with the accumulation observed when culturing the methanol-assimilating bacterium not containing the DNA of the present invention. Furthermore, the increased secretion of the L-amino acids to the outside of a cell may also be observed as decreasing intracellular concentrations of the L-amino acids when the DNA of the present invention is introduced into a methanol-assimilating bacterium.

[0028] The *Methylobacillus* bacterium of the present invention is a bacterium which belongs to the genus *Methylobacillus* and can grow using methanol as a main carbon source, and in which secretion of an L-amino acid such as L-lysine or L-arginine is facilitated by introducing the DNA of the present invention. Specific examples thereof include, but are not limited to *Methylobacillus glycogenes*, *Methylobacillus flagellatum* and so forth. Examples of *Methylobacillus glycogenes* include, but are not limited to the T-11 strain (NCIMB 11375), ATCC 21276 strain, ATCC 21371 strain, ATR80 strain (see Appl. Microbiol. Biotechnol., 42, 67-72 (1994)), A513 strain (see Appl. Microbiol. Biotechnol., 42, 67-72 (1994)) and so forth. The *Methylobacillus glycogenes* NCIMB 11375 strain is available from the National Collections of Industrial and Marine Bacteria (NCIMB Lts., Torry Research Station, 135, Abbey Road, Aberdeen AB9 8DG, United Kingdom). Examples of *Methylobacillus flagellatum* include, but are not limited to the KT strain (see Arch. Microbiol., 149, 441-446 (1988)) and so forth.

[0029] The *Methylobacillus bacterium* of the present invention can be obtained by introducing a DNA encoding a variant of a protein having a loop region and six hydrophobic helixes which is involved in secretion of L-lysine to the outside of a cell, whereby the DNA has a mutation which results in deletion of the loop region, and/or results in the protein variant substantially consisting of only the hydrophobic helixes. The expression "substantially consisting of only the hydrophobic helixes" means that the

mutant LysE is completely deficient in the loop region or deficient in most of the loop region to such an extent that the function of the mutant LysE should not be affected.

[0030] One of the embodiments of the *Methylobacillus* bacterium of the present invention is a *Methylobacillus* bacterium into which the DNA designated as *lysE*24 described in the examples has been introduced. The gene *lysE*24 is a mutant gene isolated from *Brevibacterium lactofermentum* 2256 strain, and is a homologue of the known *lysE* gene from *Corynebacterium glutamicum* R127 strain . Therefore, the DNA which is introduced into the *Methylobacillus* bacterium of the present invention may also be referred to as a "mutant *lysE*" for convenience.

[0031] The LysE protein that is encoded by the *lysE* gene has six hydrophobic helix regions. Some of these hydrophobic regions are estimated to be transmembrane domains. It is also estimated that a region between the third and fourth regions from the N-terminus is hydrophilic and has a loop structure. In the present invention, this hydrophilic region is called a loop region. The nucleotide sequence of wild-type *lysE* and the amino acid sequence of the LysE protein derived from *Brevibacterium lactofermentum* 2256 strain are shown in SEQ ID NOS: 7 and 8, respectively. In this amino acid sequence, the hydrophobic helix regions correspond to the amino acid numbers 5-20, 37-58, 67-93, 146-168, 181-203 and 211-232. The loop region corresponds to the amino acid numbers 94-145.

[0032] The inventors of the present invention found that the *lysE* gene was lethal in a methanol-assimilating bacterium, but that a DNA encoding a variant of the LysE protein that did not have the loop region or substantially consisted of only the hydrophobic helixes, increased the secretion of L-lysine and/or L-arginine to the outside of a cell of methanol-assimilating bacterium. The DNA of the present invention encodes such a

mutant LysE protein that does not have the aforementioned loop region, or that substantially consists of only the hydrophobic helixes.

[0033] The aforementioned mutant LysE is not particularly limited so long as it has one or more hydrophobic helixes and when expressed results in increased secretion of L-lysine, L-arginine or both when it is introduced into a methanol-assimilating bacterium. Specifically, a DNA encoding a mutant LysE that has all of the first to sixth hydrophobic helixes from the N-terminus is encompassed. More specifically, a DNA encoding a peptide containing the first to third hydrophobic helixes relative to the N-terminus, and encoding a peptide containing the fourth to sixth hydrophobic helixes relative to the N-terminus is encompassed. The aforementioned *lysE*24 is an example of the mutant *lysE* that encodes a peptide containing the first to third hydrophobic helixes and a peptide containing the fourth to sixth hydrophobic helixes. The *lysE*24 gene is introduced by a mutation with a stop codon downstream from the region encoding the third hydrophobic helix. When a region downstream from this stop codon was deleted as described in the examples, the mutant *lysE*24 gene did not cause L-lysine to accumulate in the medium when expressed in *Methylobacillus glycogenes* NCIMB 11375 strain. Therefore, it is estimated that a peptide containing the first to third hydrophobic helixes and a peptide containing the fourth to sixth hydrophobic helixes are separately translated and function in *Methylobacillus glycogenes*. The results show that introduction of the *lysE*24 gene into a *Methylobacillus* bacterium will result in improvement of the production of L-lysine or L-arginine.

[0034] Any microorganism can be used to generate a DNA encoding a protein involved in secretion of L-lysine to the outside of a cell, i.e., the *lysE* gene or its homologous gene, so long as it has a variant of the gene that can express the L-lysine secretion activity in a

methanol-assimilating bacterium. Specifically, coryneform bacteria such as *Corynebacterium glutamicum* (*Brevibacterium lactofermentum*), *Escherichia* bacteria such as *Escherichia coli*, *Pseudomonas* bacteria such as *Pseudomonas aeruginosa*, *Mycobacterium* bacteria such as *Mycobacterium tuberculosis* and so forth are emcompassed.

[0035] Examples of the homologous gene of *lysE* include a DNA coding for a protein which is hybridizable under stringent conditions with a probe having the nucleotide sequence of SEQ ID NO: 7 or a part thereof, and encodes a protein exhibiting the function of the *LysE* protein in a methanol-assimilating bacterium as a result of the aforementioned amino acid substitution. The aforementioned "stringent conditions" include conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of 80% or more, preferably 90% or more, more preferably 95% or more, are hybridized with each other, whereas DNAs having homology lower than the above do not hybridize with each other. Alternatively, stringent conditions are exemplified by conditions whereby DNAs hybridize with each other at a salt concentration upon ordinary conditions of washing in Southern hybridization, i.e., 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C.

[0036] A partial sequence of the nucleotide sequence of SEQ ID NO: 7 can also be used as the probe. Such a probe can be prepared by PCR using oligonucleotides based on the nucleotide sequence of SEQ ID NO: 7 as primers and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 7 as a template. When a DNA fragment having a

length of about 300 bp is used as the probe, the washing conditions of hybridization can be, for example, 2 x SSC, 0.1% SDS at 50°C.

[0037] In order to enhance the amino acid secretion gene expression in a *Methylophilus* bacterium, the gene fragment containing a *lysE* gene is ligated to a vector which is able to function in the *Methylophilus* bacterium, preferably a multi-copy type vector, to prepare a recombinant DNA which is then used to transform a host such as a *Methylophilus* bacterium. Alternatively, the gene can be incorporated into a transposon and introduced into the chromosome. Furthermore, a promoter that induces potent transcription in a *Methylophilus* bacterium can be ligated upstream from the gene.

[0038] The reference WO97/23597 discloses *lysE*, and only shows the *lysE* gene of coryneform bacterium introduced into a coryneform bacterium. Furthermore, it only mentions L-lysine as the secreted amino acid, and discloses a novel protein secretion system, including LysE having a structure containing six transmembrane helixes. However, the inventors of the present invention confirmed that LysE derived from coryneform bacteria did not function at all in methanol-assimilating bacteria.

[0039] Furthermore, the obtained factor is a novel L-lysine secretion factor, which has a basic structure different from the known LysE of coryneform bacteria havine six transmembrane helixes on one polypeptide, and this factor can no way be anticipated from the disclosure of the aforementioned patent specification that discloses LysE.

Methylobacillus Bacterium of the Present Invention

[0040] The *Methylobacillus* bacterium of the present invention is introduced with the DNA of the present invention which is able to be expressed, and has an ability to produce L-lysine or L-arginine. It can be obtained by introducing the DNA of the present

invention into a *Methylobacillus* bacterium that has the L-lysine or L-arginine producing ability. The *Methylobacillus* bacterium of the present invention can also be obtained by imparting a L-lysine or L-arginine producing ability to a *Methylobacillus* bacterium introduced with the DNA of the present invention. The *Methylobacillus* bacterium of the present invention may also be obtained by imparting a L-lysine or L-arginine producing ability by introduction of the DNA of the present invention which is able to be expressed.

[0041] A *Methylobacillus* bacterium having the L-lysine or L-arginine producing ability can be obtained by imparting a L-lysine or L-arginine producing ability to a wild-type strain of a *Methylobacillus* bacterium. Methods conventionally used for breeding of coryneform bacteria, *Escherichia* bacteria, and so forth can be used to impart the L-lysine or L-arginine producing ability. For example, such methods include, but are not limited to acquisition of auxotrophic mutant strains, analogue resistant strains or metabolic regulation mutant strains, creation of recombinant strains in which an L-lysine or L-arginine biosynthesis system enzyme is enhanced (see "Amino Acid Fermentation", the Japan Scientific Societies Press [Gakkai Shuppan Center], 1st Edition, published on May 30, 1986, pp.77 to 100) and so forth. Properties of auxotrophy, analogue resistance, metabolic regulation mutation and so forth may be individually imparted or two or more may be imparted in combination when breeding L-lysine or L-arginine producing bacteria. The biosynthesis system enzyme may be individually enhanced or two or more of them may be enhanced in combination. Furthermore, the impartation of the properties including auxotrophy, analogue resistance, metabolic regulation mutation and so forth may be combined with the enhancement of biosynthesis system enzyme.

[0042] For example, L-lysine producing bacteria can be bred to be auxotrophic for L-

homoserine or L-threonine and L-methionine (Japanese Patent Publication Nos. 48-28078 and 56-6499), or be auxotrophic for inositol or acetic acid (Japanese Patent Laid-open Nos. 55-9784 and 56-8692), or be resistant to oxalysine, lysine hydroxamate, S-(2-aminoethyl)-cysteine, γ-methyllysine, α-chlorocaprolactam, DL-α-amino-ε-caprolactam, α-amino-lauryllactam, aspartic acid analogue, sulfa drug, quinoid or N-lauroylleucine.

[0043] L-arginine producing bacteria can be bred to be resistant to a certain agent, for example, sulfa drug, 2-thiazolealanine, α-amino-β-hydroxyvaleric acid or the like; to be auxotrophic for L-histidine, L-proline, L-threonine, L-isoleucine, L-methionine or L-tryptophan in addition to resistance to 2-thiazolealanine (Japanese Patent Laid-open No. 54-44096); to be resistant to ketomalonic acid, fluoromalonic acid or monofluoroacetic acid (Japanese Patent Laid-open No. 57-18989); to be resistant to argininol (Japanese Patent Laid-open No. 62-24075); to be resistant to X-guanidine (X represents a derivative of fatty acid or aliphatic chain, Japanese Patent Laid-open No. 2-186995); to be resistant to 5-azauracil, 6-azauracil, 2-thiouracil, 5-fluorouracil, 5-bromouracil, 5-azacytosine, 6-azacytosine and so forth; to be resistant to arginine hydroxamate and 2-thiouracil; to be resistant to arginine hydroxamate and 6-azauracil (seeJapanese Patent Laid-open No. 57-150381); to be resistant to a histidine analogue or tryptophan analogue (seeJapanese Patent Laid-open No. 52-114092); to be auxotrophic for at least one of methionine, histidine, threonine, proline, isoleucine, lysine, adenine, guanine and uracil (or uracil precursor) (see Japanese Patent Laid-open No. 52-99289); to be resistant to arginine hydroxamate (see Japanese Patent Publication No. 51-6754); to be auxotrophic for succinic acid or resistant to a nucleic acid base analogue (Japanese Patent Laid-open No. 58-9692); to be deficient in the ability to metabolize arginine and to be resistant to an arginine antagonist and canavanine and auxotorophic for lysine (seeJapanese Patent Laid-

open No. 52-8729); to be resistant to arginine, arginine hydroxamate, homoarginine, D-arginine and canavanine, or resistant to arginine hydroxamate and 6-azauracil (seeJapanese Patent Laid-open No. 53-143288); to be resistant to canavanine (seeJapanese Patent Laid-open No. 53-3586) and so forth.

[0044] Hereinafter, methods for imparting or enhancing L-amino acid producing ability by enhancing an L-amino acid biosynthetic enzyme gene are exemplified.

[0045] L-lysine producing ability can be imparted by, for example, enhancing the activities of dihydrodipicolinate synthase and aspartokinase. The activities of dihydrodipicolinate synthase and aspartokinase in a *Methylophilus* bacterium can be enhanced by transforming a host such as *Methylophilus* bacterium with a recombinant DNA prepared by ligating a gene fragment encoding dihydrodipicolinate synthase and a gene fragment encoding aspartokinase with a vector that functions in the *Methylophilus* bacterium, preferably a multiple copy type vector.   The increase in copy numbers of the genes encoding dihydrodipicolinate synthase and aspartokinase in the transformant strain results in an enhancement in the activities of these enzymes.   Hereinafter, dihydrodipicolinate synthase, aspartokinase and aspartokinase III are also referred to as DDPS, AK and AKIII, respectively.

[0046] Any microorganism may provide the genes which encode DDPS and AK, so long as the chosen microorganism can express DDPS and AK activity in a *Methylobacillus*. Such microorganisms may be wild-type strains, or mutant strains derived therefrom. Specifically, examples of such microorganisms include *E. coli* (*Escherichia coli*) K-12 strain, *Methylobacillus glycogenes* NCIMB 11375 and so forth.   Since nucleotide sequences for the genes encoding DDPS (dapA, Richaud, F. et al., J. Bacteriol., 297 (1986)) and AKIII (lysC, Cassan, M., Parsot, C., Cohen, G.N. and Patte, J.C., J. Biol.

Chem., 261, 1052 (1986)) are known, these genes can be obtained by PCR using primers synthesized based on the nucleotide sequences of these genes, and using chromosomal DNA of microorganism such as *E. coli* K-12 as a template. Specific examples include, but are not limited to *dapA* and *lysC* derived from *E. coli*, as explained herein.

[0047] Preferably, the DDPS and AK used for the present invention will not be subject to feedback inhibition by L-lysine. It is known that wild-type DDPS derived from *E. coli* is subject to feedback inhibition by L-lysine (see US Patents 5,661,012 and 6,040,160), and that wild-type AKIII derived from *E. coli* is subject to suppression and feedback inhibition by L-lysine. Therefore, *dapA* and *lysC* preferably encode for DDPS and AKIII, respectively, each of which contain a mutation that eliminates the feedback inhibition by L-lysine upon introduction into a *Methylophilus* bacterium. Hereinafter, DDPS which contains a mutation that eliminates the feedback inhibition by L-lysine may also be referred to as "mutant DDPS," and a DNA encoding the mutant DDPS may also be referred to as "mutant dapA," or "dapA*." AKIII derived from *E. coli* which contains a mutation that eliminates the feedback inhibition by L-lysine may also be referred to as "mutant AKIII," and a DNA encoding the mutant AKIII may also be referred to as "mutant lysC".

[0048] However, it is not always necessary that DDPS and AK be mutated in the present invention . It is known that, for example, DDPS derived from *Corynebacterium* does not suffer feedback inhibition by L-lysine (see Korean Patent Publication No. 92-8382, US Patents 5,661,012 and 6,040,160).

[0049] A nucleotide sequence of wild-type dapA derived from *E. coli* is exemplified in SEQ ID NO: 13, and the amino acid sequence of wild-type DDPS encoded by the nucleotide sequence is exemplified in SEQ ID NO: 14.

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[0050] The DNA encoding mutant DDPS that does not suffer feedback inhibition by L-lysine may be a DNA encoding DDPS having the amino acid sequence including replacing the histidine residue at position 118 with a tyrosine residue. Furthermore, the DNA encoding mutant AKIII that does not suffer feedback inhibition by L-lysine may be a DNA encoding AKIII having the amino acid sequence including replacing the threonine residue at position 352 with an isoleucine residue (see US Patents 5,661,012 and 6,040,160).

[0051] The plasmid used for gene cloning may be any plasmid so long as it can replicate in microorganisms such as *Escherichia* bacteria. Specifically, examples of such plasmids include pBR322, pTWV228, pMW119, pUC19 and so forth.

[0052] Vectors that function in *Methylobacillus* bacteria include, for example, a plasmid that can autonomously replicate in *Methylobacillus* bacteria. Specifically, examples include RSF1010, which is a broad host spectrum vector, and derivatives thereof, for example, pAYC32 (Chistorerdov, A.Y., Tsygankov, Y.D. Plasmid, 16, 161-167 (1986)), pMFY42 (Gene, 44, 53 (1990)), pRP301, pTB70 (Nature, 287, 396, (1980)) and so forth.

[0053] To prepare a recombinant DNA via ligation of *dapA* and *lysC* to a vector that functions in *Methylobacillus* bacteria, the vector is digested with a restriction enzyme suitable for the terminus of a DNA fragment containing *dapA* and *lysC*. The ligation is usually performed by using a ligase such as T4 DNA ligase. The genes *dapA* and *lysC* may be individually incorporated into separate vectors or the same vector.

[0054] A broad host spectrum plasmid RSFD80 is known (WO95/16042), and may be used in the present invention as the plasmid having a mutant *dapA* encoding a mutant DDPS and mutant *lysC* encoding a mutant AKIII. An *E. coli* JM109 strain transformed with this plasmid was designated as AJ12396, and deposited at the National Institute of

Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary) on October 28, 1993 and received an accession number of FERM P-13936. Then, the deposit was converted to an international deposit under the provisions of the Budapest Treaty on November 1, 1994, and received an accession number of FERM BP-4859. RSFD80 can be obtained from the AJ12396 strain by a known method.

[0055] Any method can be used to introduce a recombinant DNA prepared as described above into a *Methylophilus* bacterium, so long as it provides sufficient transformation efficiency. For example, electroporation can be used (Canadian Journal of Microbiology, 43, 197 (1997)).

[0056] The DDPS and AK activities can also be enhanced by the presence of multiple copies of *dapA* and *lysC* on chromosomal DNA of a *Methylobacillus* bacterium. Multiple copies of *dapA* and *lysC* may be introduced into the chromosomal DNA of a *Methylobacillus* bacterium by homologous recombination. This can be performed by targeting a sequence present on chromosomal DNA in multiple copy number. A repetitive DNA or an inverted repeat present at the end of a transposable element can be used as the sequence present on the chromosomal DNA in a multiple copy number. Alternatively, as disclosed in Japanese Patent Laid-open No. 2-109985, multiple copies of dapA and/or lysC can be introduced into the chromosomal DNA by incorporating them into a transposon and transferring it. In both of the methods, activities of DDPS and AK will be amplified as a result of increased copy numbers of *dapA* and *lysC* in transformant strains.

[0057] Besides the above gene amplification methods, the DDPS activity and AK activity can be amplified by replacing expression control sequences, such as promoters of *dapA* and *lysC*, with stronger ones (see Japanese Patent Laid-open No. 1-215280). Examples of such strong promoters are known, and include, for example, lac promoter, trp promoter, trc promoter, tac promoter, P_R promoter and P_L promoter of lambda phage, tet promoter, amyE promoter, spac promoter and so forth. Use of these strong promoters enhances expression of *dapA* and *lysC*, and thus DDPS activity and AK activity will be amplified. Such gene expression enhancement methods can be combined with the with the gene amplification (increasing the copy number of dapA and lysC) methods described above.

[0058] Preparation of a recombinant DNA can be accomplished by ligating a gene fragment and a vector once the vector is digested with a restriction enzyme corresponding to the terminus of the gene fragment. Ligation is usually performed by ligase such as T4 DNA ligase. The usual methods well known to those with skill in the art can be used as methods for digestion, ligation of DNA, preparation of chromosomal DNA, PCR, preparation of plasmid DNA, transformation, design of oligonucleotides used as primers and so forth. Such methods are described in Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989) and so forth.

[0059] In addition to the enhancement of DDPS and AK gene expression or activity, other enzymes involved in the L-lysine biosynthesis may also be enhanced. Such enzymes include diaminopimelate pathway enzymes such as dihydrodipicolinate reductase, diaminopimelate decarboxylase, diaminopimelate dehydrogenase (see WO96/40934 for all of the foregoing enzymes), phosphoenolpyruvate carboxylase

(Japanese Patent Laid-open No. 60-87788), aspartate aminotransferase (Japanese Patent Publication No. 6-102028), diaminopimelate epimerase and aspartate semialdehyde dehydrogenase, aminoadipate pathway enzymes such as homoaconitate hydratase and so forth.

[0060] Aspartokinase, aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase, dihydrodipicolinate reductase and diaminopimelate decarboxylase derived from *Methylophilus methylotrophus* as a methanol-assimilating bacterium are disclosed in WO 00/61723.

[0061] Furthermore, the microorganisms of the present invention may have decreased activity of an enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway for L-lysine, or may be deficient in such an enzyme.   Illustrative examples of the enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway for L-lysine include homoserine dehydrogenase (see WO95/23864).

[0062] The aforementioned techniques for enhancing activities of enzymes involved in the L-lysine biosynthesis can be similarly used for L-arginine.

[0063] L-arginine producing ability can be improved by enhancing acetylornithine deacetylase activity, N-acetylglutamate-γ-semialdehyde dehydrogenase activity, N-acetyl glutamokinase activity and argininosuccinase activity (Japanese Patent Publication No. 5-23750).

[0064] L-arginine producing ability can also be improved by enhancing activity of glutamate dehydrogenase (EP 1 057 893 A1), argininosuccinate synthase (EP0 999 267 A1), carbamoyl phosphate synthetase (EP1 026 247 A1) or N-acetylglutamate synthase (see Japanese Patent Laid-open No. 57-5693) or by disrupting the gene encoding an

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arginine repressor (*argR*).

Production of L-lysine or L-arginine

[0065]   L-lysine or L-arginine can be produced by culturing a *Mehylobaccilus*

bacterium   having L-lysine or L-arginine producing ability.   L-lysine or L-arginine can

be obtained as described above from a medium upon production and accumulation.   L-

lysine or L-arginine can then be collected from the culture.

[0066] The microorganism used for the present invention can be cultured by a method

typically used in culture of a methanol-assimilating microorganism.   The medium used

for the present invention may be either a natural or synthetic medium so long as it

contains a carbon source, nitrogen source, inorganic ions and other trace amount organic

components as required.

[0067] If methanol is used as a main carbon source, L-lysine or L-arginine can be

produced at a low cost.   When methanol is used as a main carbon source, it is added to a

medium in an amount of between 0.001 to 30%.   As the nitrogen source, ammonium

sulfate or the like is used by adding it to the medium.   In addition to these, trace amount

components such as potassium phosphate, sodium phosphate, magnesium sulfate, ferrous

sulfate, manganese sulfate and so forth, can be added in small amounts.

[0068] The culture is usually performed under aerobic conditions by shaking, or aeration

by stirring, or the like at a pH of between 5 to 9, and a temperature of between 20 to

45°C, and it is typically complete within 24 to 120 hours.

[0069] Collection of L-lysine or L-arginine can usually be collected from culture by a

combination of ion exchange resin method, precipitation method, and other known

methods.

Examples

[0070] Hereafter, the present invention will be explained more specifically with reference to the following examples.

[0071] The reagents used in the following examples were obtained from Wako Pure Chemicals or Nacalai Tesque, unless otherwise indicated. The compositions of the media used in each example are shown below. pH was adjusted with NaOH or HCl for all of the media.

LB medium:

Trypton peptone (Difco)   10 g/L

Yeast extract (Difco)   5 g/L

NaCl   10 g/L

pH 7.0

These were steam-sterilized at 120°C for 20 minutes.

LB agar medium:

LB medium

Bacto agar   15 g/L

These were steam-sterilized at 120°C for 20 minutes.

SEII medium (see Journal of General Microbiology (1989) 125, 135, 3153-3164, Silman N. J., Carver M. A. & Jones C. W.; A part of the composition was modified.):

K_2HPO_4 1.9 g/L

NaH_2PO_4	1.56 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
$(NH_4)_2SO_4$	5 g/L
$CuSO_4 \cdot 5H_2O$	5 µg/L
$MnSO_4 \cdot 5H_2O$	25 µg/L
$ZnSO_4 \cdot 7H_2O$	23 µg/L
$CaCl_2 \cdot 2H_2O$	72 mg/L
$FeCl_3 \cdot 6H_2O$	9.7 mg/L
$CaCO_3$ (Kanto Kagaku)	30 g/L
Methanol	2% (vol/vol)

pH 7.0

Except for methanol, the components were steam-sterilized at 121°C for 15 minutes.

After the components were sufficiently cooled, methanol was added.

SEII agar medium:

K_2HPO_4	1.9 g/L
NaH_2PO_4	1.56 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
$(NH_4)_2SO_4$	5 g/L
$CuSO_4 \cdot 5H_2O$	5 µg/L
$MnSO_4 \cdot 5H_2O$	25 µg/L
$ZnSO_4 \cdot 7H_2O$	23 µg/L
$CaCl_2 \cdot 2H_2O$	72 mg/L
$FeCl_3 \cdot 6H_2O$	9.7 mg/L

Methanol   0.5% (vol/vol)

pH 7.0

Bacto agar (Difco)   15 g/L

Except for methanol, the components were steam-sterilized at 121°C for 15 minutes.

After the components were sufficiently cooled, methanol was added.

Example 1

[0072] Introduction of *lysE* gene derived from *Brevibacterium* bacterium into

Methylophilus bacterium

[0073] A *lysE* gene, which was a homologous gene of the gene facilitating secretion of

L-lysine known for *Corynebacterium glutamicum* R127, was cloned from a

Brevibacterium lactofermentum 2256 strain, and expression was attempted in a

Methylophilus bacterium.

[0074] (1) Construction of pRS*lysE*

[0075] In order to introduce *lysE* into a *Methylophilus* bacterium, a known plasmid pRS

(see International Patent Publication in Japanese (Kohyo) No. 3-501682) was used to

construct a plasmid pRS*lysE* for expression of *lysE*.   pRS is a plasmid having the vector

segment of the pVIC40 plasmid (International Patent Publication WO90/04636,

International Patent Publication in Japanese No. 3-501682) and obtained from pVIC40 by

deleting a DNA region encoding the threonine operon contained in the plasmid.   The

plasmid pVIC40 is derived from a broad host spectrum vector plasmid pAYC32

(Chistorerdov, A.Y., Tsygankov, Y.D., Plasmid, 1986, 16, 161-167), which is a

derivative of RSF1010.

[0076] First, a plasmid pRStac having the *tac* promoter was constructed from pRS

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according to the scheme shown in Fig. 1. The pRStac plasmid was constructed as

follows. The pRS vector was digested with restriction enzymes EcoRI and PstI and

added to a phenol/chloroform solution and mixed to terminate the reaction. After the

reaction mixture was centrifuged, the upper layer was collected, and DNAs were

collected by ethanol precipitation and separated on 0.8% agarose gel. A DNA fragment

of 8 kilobase pairs (hereinafter, "kbp") was collected by using EASY TRAP Ver. 2 (DNA

collection kit, Takara Shuzo). Alternatively, the *tac* promoter region was amplified by

PCR using the pKK223-3 plasmid (expression vector, Pharmacia) as a template and the

primers shown in SEQ ID NOS: 1 and 2 (a cycle consisting of denaturation at 94°C for

20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60

seconds was repeated for 30 cycles). Pyrobest DNA polymerase (Takara Shuzo) was

used for PCR. The DNA fragment containing the amplified *tac* promoter was purified

by using PCR prep (Promega) and then digested at the restriction enzyme sites

preliminarily designed in the primers, i.e., at EcoRI and EcoT22I sites. Then, the

reaction mixture was added to a phenol/chloroform solution and mixed to terminate the

reaction. After the reaction mixture was centrifuged, the upper layer was collected and

DNAs were collected by ethanol precipitation and separated on 0.8% agarose gel. A

DNA fragment of about 0.15 kbp was collected by using EASY TRAP Ver. 2.

[0077] The digestion product of the pRS vector and the *tac* promoter region fragment

prepared as described above were ligated by using DNA Ligation Kit Ver. 2 (Takara

Shuzo). This ligation reaction solution was used to transform *Escherichia coli* (*E. coli*

JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium

containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies that

appeared on the agar medium were each inoculated into LB liquid medium containing 20

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mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkali-SDS method and structure of each plasmid was confirmed by digestion with restriction enzymes to obtain pRStac. A plasmid in which the transcription directions of the streptomycin resistance gene on the pRS vector and the *tac* promoter were identical to each other was selected as pRStac.

[0078] pRStac obtained as described above was digested with Sse8387I (Takara Shuzo) and SapI (New England Biolabs), added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNAs were collected by ethanol precipitation and separated on 0.8% agarose gel to obtain a DNA fragment of about 9.0 kbp.

[0079] The *lysE* gene fragment was also amplified by PCR using chromosome extracted from the *Brevibacterium lactofermentum* 2256 strain (ATCC13869) as a template and the primers shown in SEQ ID NOS: 5 and 6 (denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 90 seconds). Pyrobest DNA polymerase (Takara Shuzo) was used for PCR. To enable expression of the *lysE* gene in a *Methylophilus* bacterium, the primers were designed so that nucleotides of 9-15 bp from the translation initiation codon of the *lysE* gene should be replaced with a sequence that is known to function in a *Methylophilus* bacterium (Wyborn, N.R., Mills, J., Williamis, S.G. and Jones, C.W., Eur. J. Biochem., 240, 314-322 (1996)). The resulting fragment was purified by using PCR prep (Promega) and then digested with Sse8387I and SapI. The reaction mixture was added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNAs were collected by ethanol precipitation and further collected from 0.8% agarose gel.

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[0080] The digestion product of the pRStac vector and the *lysE* gene region fragment prepared as described above were ligated using DNA Ligation Kit Ver. 2 (Takara Shuzo). This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies that appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkali-SDS method and structure of each plasmid was confirmed by digestion with restriction enzymes and determination of nucleotide sequence, confirming the presence of pRS*lysE* (Fig. 1). In pRS*lysE*, the *lysE* gene was positioned so that its transcription direction is the same as that of the *tac* promoter.

[0081] (2) Introduction of pRS*lysE* into *Methylophilus* bacterium

[0082] pRS*lysE* obtained as described above was introduced into *Methylophilus methylotrophus* AS1 strain (NCIMB10515) by electroporation (Canadian Journal of Microbiology, 43, 197 (1997)). In addition, pRS was also introduced into the AS1 strain as a control in the same manner as for pRS*lysE*. As a result, several thousands of colonies were obtained per 1 μg of DNA with pRS used as a control, whereas only several colonies were obtained with pRS*lysE*.

[0083] When plasmids were extracted from transformant strains estimated to be introduced with pRS*lysE* and their nucleotide sequences investigated, a spontaneous mutation was introduced in a region encoding *lysE* for all the investigated plasmids, and in some cases, a nonsense mutation was introduced as the mutation, by which a codon encoding an amino acid was replaced with a stop codon that terminated the translation.

In other plasmids, deletion was observed in the *lysE* gene. Iin either case, the function of *lysE* in pRS*lysE* was lost. However, when a plasmid was prepared in which a part of the region encoding *lysE* was intentionally deleted so that the function of the *lysE* gene is eliminated (pRS*lysE*Δ1) and introduced into *Methylophilus methylotrophus*, it could be introduced at a frequency equivalent to that of the control pRS vector.

[0084] The aforementioned pRS*lysE*Δ1 was a plasmid in which a region from *Pvu*I site (recognizes CGATCG of the 203-209th positions in SEQ ID NO: 7) to *Mlu*I site (recognizes ACGCGT of the 485-491st positions of the same) present in the region encoding *lysE* was deleted. Specifically, pRS*lysE* was constructed by digestion with *Pvu*I and *Mlu*I (Takara Shuzo), added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNAs were collected by ethanol precipitation and separated on 0.8% agarose gel to obtain a DNA fragment of about 10 kbp. This DNA fragment was blunt-ended by using DNA Blunting Kit (Takara Shuzo). The product was self-ligated using DNA Ligation Kit Ver. 2 (Takara Shuzo).

[0085] This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies that appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkali-SDS method, and the structure of each plasmid was confirmed by digestion with restriction enzymes, to obtain the pRS*lysE*Δ1 plasmid.

[0086] As described above, the introduction frequency of pRS*lysE* carrying the full

length *lysE* gene into *Methylophilus methylotrophus* was extremely low, and only plasmids having a *lysE* mutant gene containing a mutation that eliminated the function could be introduced. Considering these facts together, it was estimated that the introduction of the *lysE* gene into *Methylophilus methylotrophus* was lethal. This indicates that the *lysE* gene cannot universally function for the secretion of L-lysine in heterogenous bacteria.

[0087] The *Methylophilus methylotrophus* AS1 strain harboring pRS*lysE* containing the above described mutation was applied to an SEII plate containing 20 mg/L of streptomycin and cultured overnight at 37°C. Then, the cells from about 0.3 cm^2 of the medium surface were scraped, inoculated into SEII production medium (20 ml) containing 20 mg/L of streptomycin, and cultured at 37°C for 34 hours with shaking. After completion of the culture, the cells were removed by centrifugation and the L-lysine concentration in the culture supernatant was determined by using an amino acid analyzer (Nihon Bunko, high speed liquid chromatography). As a result, substantially no strain was obtained in which secretion of L-lysine was enhanced in spite of introduction of the mutant *lysE* gene.

[0088] Acquisition of gene providing L-lysine secretion activity in *Methylophilus* bacteria

[0089] As described in the preceding section, the known *lysE* gene is lethal in *Methylophilus* bacteria, and as a result, many mutant genes for which function was lost were subsequently obtained.

[0090] During analysis of pRS*lysE* containing a mutation, a mutant *lysE* gene that functioned in *Methylophilus* bacteria but was not lethal was obtained.

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[0091] This mutant *lysE* gene was designated as *lysE*24 gene. When the nucleotide sequence of *lysE*24 gene was analyzed, it was found that this mutation did not result in an amino acid substitution, but a nonsense mutation introducing a stop codon around the center of the translation region of *lysE*. It has been reported that the *lysE* gene of *Corynebacterium* bacteria encodes a membrane protein having six hydrophobic helixes (Vrlijc M., Sahm H., and Eggeling L., Molecular Microbiology 22:815-826 (1996)). In contrast, it was found that since the above *lysE*24 gene contained a stop codon, the protein encoded by this gene had a structure different from that of the wild-type LysE protein. As a result, the LysE mutant functioned in *Methylophilus* bacteria due to this structure.

[0092] The nucleotide sequence of *lysE*24 and the amino acid sequence encoded by the nucleotide sequence are shown in SEQ ID NOS: 9 and 10, respectively. The nucleotide sequence of wild-type *lysE* and the amino acid sequence encoded by the nucleotide sequence are shown in SEQ IDS NO: 7 and 8, respectively. In *lysE*24, T (thymine) was inserted after G (guanine) at the 355th position of the wild-type *lysE* gene. The plasmid having *lysE*24 was designated as pRS*lysE*24 (Fig. 1). When pRS*lysE*24 was introduced anew into the AS1 strain, the plasmid could be introduced at a frequency substantially equivalent to that of pRS. In Table 1, the result of L-lysine concentration measurement for culture supernatant of the plasmid-introduced strain is shown, which measurement was performed in the same manner as above (Example 1, part (2)).

[0093] Table 1

Strain	Production amount of L-lysine (g/L)
AS1/pRS	<0.01

AS1/pRS*lysE*24	0.1

[0094] The *E. coli* JM109 strain transformed with pRS*lysE*24 was designated as AJ13830, and this strain was deposited at the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary on June 4, 2001 and received an accession number of FERM P-18369. Then, the deposit was converted to an international deposit under the provisions of the Budapest Treaty on May 13, 2002, and received an accession number of FERM BP-8040.

[0095] The AS1 strain containing a plasmid which was obtained from the *lysE*24 gene by deleting a region downstream from the stop codon generated by the aforementioned mutation, that is, the pRS*lysE*Δ1 plasmid described in Example 1, part (1), did not accumulate L-lysine in the medium. Based on this result, it is estimated that not only the first half of the peptide, but also the latter half of the peptide was expressed from the *lysE*24 gene to form a complex.

Example 2

[0096] As described above, it was found that the *lysE* gene, which is homologous to the gene that facilitates secretion of L-lysine for *Corynebacterium*, did not function in *Methylophilus* bacteria at all, whereas its variant *lysE*24 functioned in *Methylophilus* bacteria. Accordingly, whether *lysE* gene of a *Corynebacterium* and *lysE*24 obtained in Example 1 would function in a *Methylobacillus* bacterium were investigated.

[0097] (1) Construction of pRS*lysE*-Tc

[0098] In order to introduce wild-type *lysE* gene derived from a *Corynebacterium* into a

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Methylobacillus bacterium, the drug resistance marker of the pRS*lysE* constructed in Example 1 was first changed from a streptomycin resistance gene to a tetracycline resistance gene.   This is because streptomycin resistance cannot be used as a marker since *Methylobacillus* bacteria originally exhibited resistance to streptomycin.

[0099] Specifically, pRS*lysE* was first digested with the restriction enzyme EcoRI, added to a phenol/chloroform solution and mixed to terminate the digestion reaction.   After the reaction mixture was centrifuged, the upper layer solution was collected, and DNA fragments were collected by ethanol precipitation and separated by 0.8% agarose gel electrophoresis.   A DNA fragment of about 10 kbp was collected by using EASY TRAP Ver. 2 (DNA Collection Kit, Takara Shuzo).

[0100]   The tetracycline resistance gene region was amplified by PCR using pRK310 (Journal of Molecular Biology 239, 623-663 (1994)) as a template DNA and the DNA primers shown in SEQ ID NOS: 11 and 12 (a cycle consisting of denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60 seconds was repeated for 30 cycles).   Pyrobest DNA polymerase (Takara Shuzo) was used for PCR.   The DNA fragment containing the amplified tetracycline resistance gene was purified by using PCR prep (Promega) and then collected by ethanol precipitation. This fragment was further digested at the restriction sites preliminarily designed in the primers, i.e., digested at EcoRI site, and added to a phenol/chloroform solution and mixed to terminate the reaction.   Subsequently, after this reaction mixture was centrifuged, the upper layer was collected, and DNAs were collected by ethanol precipitation.   Then, the target DNA fragment was separated by 0.8% agarose gel electrophoresis to collect a DNA fragment of about 1.5 kbp.

[0101]   The tetracycline resistance gene can also be obtained in the same manner as

described above by PCR using another plasmid instead of pRK310, for example, the

pRK2 plasmid, a parent plasmid of pRK310 (available as NICMB11968 from NICMB,

see Science, 190, 1226-1228 (1975), or Plasmid, 5, 10-19 (1981)) as a template.

[0102]   The DNA fragment derived from pRS*lysE* prepared as described above and the

DNA fragment containing the tetracycline resistance gene region were ligated by using

DNA Ligation Kit Ver. 2 (Takara Shuzo).   This reaction mixture was used to transform

E. coli JM109 competent cells (Takara Shuzo).   The cells were plated on LB agar

medium containing 20 mg/L of streptomycin and 15 mg/L of tetracycline and cultured

overnight at 37°C.   The colonies that appeared on the agar medium were each

inoculated into LB liquid medium containing 20 mg/L of streptomycin and 15 mg/L of

tetracycline and cultured at 37°C for 8 hours with shaking.   Plasmid DNA was extracted

from this culture broth by the alkali-SDS method, and the structure of each plasmid was

confirmed by digestion with restriction enzymes to obtain pRS*lysE*-Tc.

[0103]   (2) Introduction of pRS*lysE*-Tc into *Methylobacillus* bacterium

[0104]   pRS*lysE*-Tc obtained as described above was introduced into the

Methylobacillus glycogenes NCIMB11375 strain by electroporation (Canadian Journal of

Microbiology, 43, 197 (1997)).   As a control, pRK310 was introduced into the

NCIMB11375 strain in the same manner as that for pRS*lysE*-Tc.   As a result, several

thousands of colonies were obtained per 1 μg of DNA with pRK310 used as a control,

whereas only several colonies were obtained with pRS*lysE*-Tc.   Thus, it was found that

the *lysE* gene derived from a *Corynebacterium* bacterium did not function in

Methylobacillus bacteria as in *Methylophilus* bacteria.

[0105]   (3) Construction of pRS*lysE*24-Tc

[0106]   Subsequently, in order to investigate whether *lysE*24 functioned in *Methylobacillus* bacteria, the drug resistance marker of the pRS*lysE*24 plasmid constructed in Example 1 was changed from the streptomycin resistance gene to a tetracycline resistance gene.

[0107]   Specifically, pRS*lysE* was first digested with a restriction enzyme EcoRI, added to a phenol/chloroform solution and mixed to terminate the digestion reaction. After the reaction mixture was centrifuged, the upper layer solution was collected, and DNA fragments were collected by ethanol precipitation and separated by 0.8% agarose gel electrophoresis.   A DNA fragment of about 10 kbp was collected by using EASY TRAP Ver. 2 (DNA Collection Kit, Takara Shuzo).

[0108]   Further, the tetracycline resistance gene fragment was obtained in the same manner as described above by amplifying the gene region from pRK310 by PCR using the primers shown in SEQ ID NOS: 11 and 12 and digesting the fragment with EcoRI.

[0109]   The DNA fragment derived from pRS*lysE*24 prepared as described above and the DNA fragment containing the tetracycline resistance gene region were ligated by using DNA Ligation Kit Ver. 2 (Takara Shuzo).   This reaction mixture was used to transform *E. coli* JM109 competent cells (Takara Shuzo).   The cells were plated on LB agar medium containing 20 mg/L of streptomycin and 15 mg/L of tetracycline and cultured overnight at 37°C.   The colonies that appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and 15 mg/L of tetracycline and cultured at 37°C for 8 hours with shaking.   Plasmid DNA was extracted from this culture broth by the alkali-SDS method, and the structure of each plasmid was confirmed by digestion with restriction enzymes, to obtain pRS*lysE*24-Tc.

[0110]   (4) Introduction of pRS*lysE*24-Tc into *Methylobacillus* bacterium

[0111]   pRS*lysE*24-Tc obtained as described above was introduced into the

Methylobacillus glycogenes NCIMB11375 strain by electroporation (Canadian Journal of

Microbiology, 43, 197 (1997)).   In addition, pRK310 was introduced into the

NCIMB11375 strain as a control.   As a result, pRS*lysE*24-Tc could be introduced at a

frequency substantially equivalent to that of the control pRK310.   The results of L-

lysine concentration measurement for culture supernatants of the plasmid-introduced

strains are shown in Table 2, which measurement was performed in the same manner as

in Example 1, part (2).

[0112]   Table 2

Strain	Production amount of L-lysine (g/L)	Production amount of L- arginine (g/L)
NCIMB11375/pRK310	< 0.01	< 0.01
NCIMB11375/pRS*lysE*24-Tc	1.57	0.16

[0113]   It was found that introduction of the *lysE*24 gene into *Methylobacillus*

glycogenes NICMB11375 strain resulted in accumulation of L-lysine in the medium.   It

was determined that this was caused by enhancement of the secretion of L-lysine.

Furthermore, when concentrations of other L-amino acids in the culture supernatant were

investigated, L-arginine was accumulated with the NCIMB11375/pRS*lysE*24-Tc strain,

and thus it was found that *lysE*24 had activity for secreting not only L-lysine but also L-

arginine.

[0114]   From the above investigation, it was found that *lysE*24 which functioned in

Methylophilus bacteria also functioned in *Methylobacillus* bacteria.

Example 3: Introduction of L-lysine biosynthesis system enzyme gene and *lysE*24 gene into *Methylobacillus glycogenes*

[0115]   It was found that when the *lysE*24 gene was introduced into *Methylobacillus glycogenes* NCIMB11375 strain, L-lysine accumulated in the medium.   It was considered that this was caused by enhancement of the secretion of L-lysine.   Therefore, the effect of introducing the *lysE*24 gene into *Methylobacillus glycogenes*   on enhancement of the L-lysine biosynthesis gene was investigated.

[0116]   <1> Construction of plasmid pRSdapA having dapA* gene

[0117]   A plasmid was prepared having a gene encoding dihydrodipicolinate synthase as an L-lysine biosynthesis system enzyme gene, that was not subject to feedback inhibition by L-lysine (dapA*).

[0118]   pRStac prepared in Example 1 was digested with Sse8387I and *Xba*I and added to a phenol/chloroform solution and mixed to terminate the reaction.   After the reaction mixture was centrifuged, the upper layer was collected, and DNAs were collected by ethanol precipitation and separated on 0.8% agarose gel to collect a DNA fragment of about 9 kbp.

[0119]   The *dapA** gene fragment was amplified by PCR using the known plasmid RSFD80 (seeWO90/16042) which contains that gene as a template and the primers shown in SEQ ID NOS: 3 and 4 (denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60 seconds).   Pyrobest DNA polymerase (Takara Shuzo) was used for PCR.   The resulting *dapA** fragment was purified using PCR prep (Promega) and then digested with restriction enzymes *Sse*8387I

and *Xba*I.   The reaction mixture was added to a phenol/chloroform solution and mixed

to terminate the reaction.   After the reaction mixture was centrifuged, the upper layer

was collected and DNAs were collected by ethanol precipitation and separated on 0.8%

agarose gel to collect a DAN fragment of about 0.1 kbp.

[0120]   The digestion product of the pRStac vector and the *dapA** gene region

fragment prepared as described above were ligated by using DNA Ligation Kit Ver. 2

(Takara Shuzo).   This ligation reaction solution was used to transform *Escherichia coli*

(*E. coli* JM109 competent cells, Takara Shuzo).   The cells were plated on LB agar

medium containing 20 mg/L of streptomycin and incubated overnight at 37°C.   The

colonies that appeared on the agar medium were each inoculated into LB liquid medium

containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking.

Plasmid DNA was extracted from each culture broth by the alkali-SDS method and the

structure of each plasmid was confirmed by digestion with restriction enzymes and

determination of nucleotide sequence, confirming the presence of pRSdapA plasmid.   In

pRSdapA plasmid, the *dapA** gene was positioned so that its transcription direction is the

same as that of the *tac* promoter.

[0121]   The *E. coli* JM109 strain transformed with the pRSdapA plasmid was

designated as AJ13831, and this strain was deposited at the independent administrative

corporation, National Institute of Advanced Industrial Science and Technology,

International Patent Organism Depositary on June 4, 2001 and received an accession

number of FERM P-18370.   Then, the deposit was converted to an international deposit

under the provisions of the Budapest Treaty on May 13, 2002, and received an accession

number of FERM BP-8041.

[0122]   <2> Construction of plasmid having *lysE*24 and *dapA**, S*lysE*24-dapA-Tc

[0123]   A plasmid having pRS*lysE*24 inserted with the *dapA** gene was constructed according to the scheme shown in Fig. 2 to evaluate the effect of combining *lysE*24 and *dapA**,

[0124]   pRS*lysE*24 prepared in Example 1 was digested with a restriction enzyme SapI and blunt-ended by using DNA Blunting Kit (Takara Shuzo).   The plasmid pRSdapA having *dapA** was digested with restriction enzymes EcoRI and SapI, and a fragment of about 1 kbp containing tac promoter and *dapA** region was separated on 0.8% agarose gel.   This fragment was collected by using EASY TRAP Ver. 2 (Takara Shuzo).   This fragment was blunt-ended as described above and ligated to the aforementioned digestion product of pRS*lysE*24 by using DNA Ligation Kit Ver. 2 (Takara Shuzo).

[0125]   The aforementioned ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo).   The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C.   The colonies that appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking.   Plasmid DNA was extracted from this culture broth by the alkali-SDS method, and the structure of each plasmid was confirmed by digestion with restriction enzymes and determination of nucleotide sequence, confirming the presence of the pRS*lysE*dapA plasmid.

[0126]   The *E. coli* JM109 strain transformed with the pRS*lysE*dapA plasmid was designated as AJ13832, and this strain was deposited at the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary on June 4, 2001 and received an accession

number of FERM P-18371.   Then, the deposit was converted to an international

deposition under the provisions of the Budapest Treaty on May 13, 2002, and received an

accession number of FERM BP-8042.

[0127]   Subsequently, the drug resistance marker gene of the pRS*lysE*dapA plasmid

was changed from a streptomycin resistance gene to a tetracycline resistance gene.

First, the pRS*lysE*dapA plasmid was digested with a restriction enzyme EcoRI, added to

a phenol/chloroform solution and mixed to terminate the digestion reaction.   After the

reaction mixture was centrifuged, the upper layer solution was collected, and DNA

fragments were collected by ethanol precipitation and separated by 0.8% agarose gel

electrophoresis.   A DNA fragment of about 11 kbp was collected by using EASY TRAP

Ver. 2 (DNA Collection Kit, Takara Shuzo).

[0128]   The tetracycline resistance gene fragment was obtained in the same manner as

in Example 1 by amplifying the gene region from pRK310 by PCR using the primers

shown in SEQ ID NOS: 11 and 12 and digesting the amplification product with EcoRI.

[0129]   The DNA fragment derived from pRS*lysE*dapA prepared as described above

and the DNA fragment containing the tetracycline resistance gene region were ligated by

using DNA Ligation Kit Ver. 2 (Takara Shuzo).   This reaction mixture was used to

transform *E. coli* JM109 competent cells (Takara Shuzo).   The cells were plated on LB

agar medium containing 20 mg/L of streptomycin and 15 mg/L of tetracycline and

cultured overnight at 37°C.   The colonies that appeared on the agar medium were each

inoculated into LB liquid medium containing 20 mg/L of streptomycin and 15 mg/L of

tetracycline and cultured at 37°C for 16 hours with shaking.   Plasmid DNA was

extracted from this culture broth by the alkali-SDS method, and the structure of each

plasmid was confirmed by digestion with restriction enzymes confirming the presence of

pRS*lysE*-dapA-Tc.

[0130]   (2) Introduction of pRS*lysE*-dapA-Tc into *Methylobacillus glycogenes*

NCIMB111375 strain and production of amino acids

[0131]   The pRS*lysE*-dapA-Tc plasmid obtained by the above method was introduced

into the *Methylobacillus glycogenes* NCIMB111375 strain by electroporation.   The

obtained transformant strain (henceforth also referred to as "NCIMB111375/pRS*lysE*-

dapA-Tc"), strain introduced with the aforementioned pRS*lysE*24-Tc (henceforth also

referred to as "NCIMB111375/pRS*lysE*-Tc") and strain introduced with pRK310 as a

control (henceforth also referred to as "NCIMB111375/pRK310") were cultured as

follows to investigate the L-amino acid concentrations in the culture supernatant.

[0132]   Each transformant strain was applied to an SEII plate containing 15 mg/L of

tetracycline and cultured at 30°C for two days.   Then, the cells on about 10 cm^2 of the

medium surface were scraped, inoculated into SEII production medium (20 ml)

containing 15 mg/L of tetracycline, and cultured at 37°C for 60 hours with shaking.

After completion of the culture, the cells were removed by centrifugation, and the L-

lysine concentration in the culture supernatant was determined by using an amino acid

analyzer (Nihon Bunko, high speed liquid chromatography).   The results are shown in

Table 3.   It was found that the amount of L-lysine that accumulated in the medium

further improved in the NCIMB/pRS*lysE*-dapA-Tc strain compared with the strain

having pRS*lysE*24-Tc.   That is, it is considered that the rate limitation regarding the

secretion was cancelled by the introduction of the *lysE*24 gene, and the *dapA** gene-

enhancing effect was exhibited in a synergistic manner.

[0133]   Table 3

Strain	Production amount of L-lysine (g/L)	Production amount of L- arginine (g/L)
NCIMB11375/pRK310	< 0.02	< 0.01
NCIMB11375/pRS*lysE*24-Tc	1.57	0.16
NCIMB11375/pRS*lysE*-dapA-Tc	1.72	0.14

[0134]   While the invention has been described in detail with reference to preferred

embodiments thereof, it will be apparent to one skilled in the art that various changes can

be made, and equivalents employed, without departing from the scope of the invention.

Each of the aforementioned documents, as well as the foreign priority document,

JP2002336340, is incorporated by reference herein in its entirety.